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(54) Title: *IN VITRO* PROPAGATION OF EMBRYONIC STEM CELLS

(57) Abstract

The present invention relates generally to the use of leukaemia inhibitory factor (LIF) in the maintenance and derivation of embryonic stem (ES) cells in culture. The ES cells are maintained and/or derived from animal embryos by culturing said cells or embryos in a culture medium containing an effective amount of LIF for a time and under conditions sufficient to maintain and/or derive said ES cells. The ES cells may be passaged in LIF and used to make chimaeric animals.

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IN VITRO PROPAGATION OF EMBRYONIC STEM CELLS

This invention relates to the use of a previously discovered and characterised molecule, leukaemia inhibitory factor (LIF), in the isolation and propagation of embryonic stem cells *in vitro*.

Embryonic stem (ES) cells, the pluripotent outgrowths of blastocysts, can be cultured and manipulated *in vitro* and then returned to the embryonic environment to contribute normally to all tissues including the germline (for review see Robertson, E.J. (1986) Trends in Genetics 2:9-13). Not only can ES cells propagated *in vitro* contribute efficiently to the formation of chimaeras, including germline chimaeras, but in addition, these cells can be manipulated *in vitro* without losing their capacity to generate germ-line chimaeras (Robertson, E.J. et.al. (1986) Nature 323:445-447).

ES cells thus provide a route for the generation of transgenic animals such as transgenic mice, a route which has a number of important advantages compared with more conventional techniques, such as zygote injection and

viral infection (Wagner and Stewart (1986) in Experimental Approaches to Embryonic Development. J. Rossant and A. Pedersen eds. Cambridge: Cambridge University Press), for introducing new genetic material into such animals. First, the gene of interest can be introduced and its integration and expression characterised *in vitro*. Secondly, the effect of the introduced gene on the ES cell growth can be studied *in vitro*. Thirdly, the characterised ES cells having a novel introduced gene can be efficiently introduced into embryos by blastocyst injection or embryo aggregation and the consequences of the introduced gene on the development of the resulting transgenic chimaeras monitored during pre- or post-natal life. Fourthly, the site in the ES cell genome at which the introduced gene integrates can be manipulated, leaving the way open for gene targeting and gene replacement (Thomas, K.R. and Capecci, M.R. (1987) Cell 51:503-512).

However, it is known that ES cells and certain EC (embryonal carcinoma) cell lines will only retain the stem cell phenotype *in vitro* when cultured on a feeder layer of fibroblasts (such as murine STO cells, e.g. Martin, G.R. and Evans, M.J. (1975) Proc. Natl. Acad. Sci. USA 72:1441-1445) or when cultured in medium conditioned by certain cells (e.g. Koopman, P. and Cotton, R.G.H. (1984) Exp. Cell Res. 154:233-242; Smith, A.G. and Hooper, M.L. (1987) Devel.Biol. 121:1-91). In the absence of feeder cells or conditioned medium, the ES cells spontaneously differentiate into a wide variety of cell types, resembling those found during embryogenesis and in the adult animal. The factors responsible for maintaining the pluripotency of ES cells have, however, remained poorly characterised.

In work leading to the present invention, it has been found that LIF has the capacity to substitute for, or be added to, feeder layers (or conditioned medium) in supporting the maintenance of pluripotential ES cells *in vitro*.

LIF is a protein that has previously been purified, cloned and produced in large quantities in purified recombinant form from both *Escherichia coli* and yeast cells. (International Patent Application No. PCT/AU88/00093, filed March 31, 1988.) LIF has been defined as a factor, the properties of which include:

1. it has the ability to suppress the proliferation of myeloid leukaemic cells such as M1 cells, with associated differentiation of the leukaemic cells; and
2. it will compete with a molecule having the defined sequence of murine LIF or human LIF (defined in International Patent Application No. PCT/AU88/00093) for binding to specific cellular receptors on M1 cells or murine or human macrophages. In addition to the biological properties previously disclosed for murine and human LIF, LIF has now been found to have the following properties:

(a) it allows the derivation and maintenance in the absence of feeder cells of the pluripotential phenotype *in vitro* of ES cells.

(b) it allows the aforementioned ES cells, after passage *in vitro* in the presence of LIF, to contribute to somatic and germline cell tissues of chimaeric animals such as mice when re-introduced into the embryonic environment;

(c) it demonstrates selective binding to high affinity receptors on murine ES (EKcs-1 (previously known as CS1) and D3) and EC (PCC3-3A and F9) cells; and

(d) specific binding of ^{125}I-LIF to high affinity receptors is not in competition with insulin, IGF-I, IGF-II, acidic and basic FGF, TGFβ, TNFα, TNFβ, NGF, PDGF, EGF, IL-1, IL-2, IL-4, GM-CSF, G-CSF, Multi-CSF nor erythropoietin, but is in competition with murine and human LIF.

Accordingly, a first aspect of the present invention relates to a method for the isolation of embryonic stem (ES) cells from animal embryos in vitro which method comprises deriving ES cells from said embryos in culture medium, said culture medium containing an effective amount of leukaemia inhibitory factor (LIF), for a time and under conditions sufficient for the development of said ES cells. The embryos used may be isolated from animals including, but not limited to, humans and a number of other animal species such as birds (eg. chickens), mice, sheep, pigs, cattle, goats and fish.

A second aspect of the present invention, contemplates a process for maintaining animal embryonic stem (ES) cells in vitro while retaining their pluripotential phenotype, which process comprises culturing said cells in a culture medium containing an effective amount of leukaemia inhibitory factor (LIF) under conditions sufficient to maintain said cells. The ES cells in accordance with this aspect of the invention include cells from humans, mice, birds (eg. chickens), sheep, pigs, cattle, goats and fish.

The LIF used in the culture medium is preferably recombinant LIF produced, by way of example, in accordance with the methods described in International Patent Application No. PCT/AU88/00093. In accordance with the present invention, it has been found that recombinant LIF and in particular recombinant human and murine LIF are effective substitutes for, or additives to, feeder layers

or conditioned medium in maintaining ES cells *in vitro*. For the purposes of the present description recombinant LIF is produced in *E. coli* and yeast using the methods described in International Patent Application No. PCT/AU88/00093, however, it is within the scope of the present invention to include recombinant LIF produced in other hosts including mammalian and insect cells and to synthetic LIF.

In another aspect, the present invention extends to ES cells derived from animal embryos by passage in a culture medium containing LIF, to such ES cells having additional genetic material inserted therein, and to chimaeric animals such as chimaeric mice or transgenic progeny of said animals generated by known techniques using ES cells which have been maintained *in vitro* in a LIF-containing culture medium.

Thus, the invention extends to the generation and maintenance of ES cells from humans, mice, birds (eg. chickens), sheep, pigs, cattle, goats and fish and to the generation of transgenic chimaeric animals and their transgenic progeny using the ES cells isolated from animal species such as mice, birds (eg. chickens), sheep, pigs, cattle, goats and fish. This invention also includes the use of LIF in culture media to modulate the survival and growth of human and other animal species such as cattle germ cells and embryonic cells, for example, for use in *in vitro* fertilisation and other procedures.

The present invention may also be described by reference to the following figures:

Figure 1 is a graphical representation showing the effect on ES cells of different concentrations of LIF.

Figure 2 is a pictorial representation showing ES cell morphology in the presence and absence of LIF.

Figure 3 is a graphical (A and C) and pictorial (B) representation showing the binding of ^{125}I-LIF to ES cells (EKcs-1) and EC cells (F9 and PCC3-A).

The present invention is directed to a method for the isolation and maintenance of embryonic stem (ES) cells from animal embryos _in vitro_ which method comprises deriving and/or maintaining said ES cells from said embryos in culture medium containing an effective amount of leukaemia inhibitory factor (LIF), for a time and under conditions sufficient for the derivation and/or maintenance of said ES cells. The animal embryos may be isolated from a number of animal species such as humans, mice, birds (eg. chickens), sheep, pigs, cattle, goats and fish. By reference herein to "animal embryos" includes reference to "animal blastocysts". Furthermore, the present invention is exemplified using human LIF with murine ES cells (heterologous system) and murine LIF with murine ES cells (homologous system). This is done with the understanding that the present invention contemplates LIF from any animal species in heterologous or homologous systems with animal embryos from animal species such as humans, mice, birds (e.g. chickens), sheep, pigs, cattle, goats and fish. Although in certain circumstances, a heterologous system will work effectively, it may be preferable to use homologous systems. Given the teachings herein, it will be routine for the skilled technician to ascertain whether a homologous or heterologous system is required in order to isolate or maintain particular animal ES cells.

By "culture medium" is meant a suitable medium capable of supporting growth of ES cells. Examples of suitable culture media useful in practicing the present invention are Eagles medium or modifications or equivalents thereof such as Dulbecco's or Glasgows

modified Eagle's medium with supplements such as 5% - 30% (v/v) foetal calf serum and where necessary 0.01 to 1.0 mM ß-mercaptoethanol but preferably about 0.1 mm ß-mercaptoethanol. The culture medium may or may not contain feeder cells and LIF may be used to substitute for, or add to, said feeder cells. When required, LIF, or more particularly synthetic or recombinant LIF, is added to the medium at a concentration of about 100 - 1,000,000 units/ml and preferably about 100 - 100,000 units/ml and even more preferably 500 - 10,000 units/ml where 50 units are defined as the amount of LIF which in one millilitre induces a 50% reduction in clone formation by murine M1 myeloid cells. By "recombinant LIF" is meant the LIF prepared by genetic engineering means such as, for example, according to International Patent Application No. PCT/AU88/00093 where a number of hosts such as bacteria (eg. _E. coli_) or yeast cells may be employed. In accordance with the present invention, the effective derivation time is from 1 day to 20 weeks and particularly from 1 to 8 weeks.

Another aspect of the present invention contemplates a process for maintaining animal ES cells _in vitro_ while retaining their pluripotential phenotype which process comprises culturing said cells in a culture medium containing an effective amount of LIF under conditions sufficient to maintain said cells. The ES cells in accordance with this aspect of the invention include cells derived from humans, mice, birds (eg. chickens), sheep, pigs, cattle, goats and fish. As with the isolation of ES cells from animal embryos, the LIF used in the aformentioned process is preferably recombinant LIF. The culture medium may or may not contain feeder cells.

In accordance with the present invention, "pluripotential cells" and "embryonic stem cells" are those which retain the developmental potential to differentiate into all somatic and germ cell lineages.

The ability of recombinant LIF to maintain the stem cell phenotype of ES cells is demonstrated by transferring ES cells D3 and HD5 into normal cell culture medium in the presence of varying concentrations of purified yeast-derived recombinant human LIF (rY-HLIF) or E. coli - derived recombinant mouse LIF (rE-MLIF). At concentrations of 1000 - 5000 units/ml of rY-HLIF or rE-MLIF more than 90% of the D3 and HD5 ES cells retained their stem cell phenotype. In contrast, the ES cells maintained in normal culture medium differentiated over a period of 3 - 6 days. The proportion of colonies having the stem cell phenotype was related to the concentration of LIF in the culture medium. In addition to maintaining established ES cell lines, six new ES cell lines (MBL-1,2,3,4,5 & 6) were isolated from blastocysts in the absence of feeder cells when the media was supplemented with 1000 units/ml rE-HLIF. Long term maintenance of the ES cell lines D3, HD5 and MBL-1 to 6 in LIF for up to 22 passages (approximately 100 cell generations or 10 weeks) did not noticeably alter the growth characteristics of these ES cells or their dose dependency on LIF. The ability of these ES cells to differentiate into all somatic and germ cell linages was confirmed by reintroduction of D3 and MBL-1 cells into blastocysts. Approximately 50% of the progeny analysed contained tissues derived from the injected ES cells with levels of overt chimaerism as high as 90% in individual mice. To test for germline transmission of ES derived cells male chimaeras were mated to C57BL/6J mice. Three D3 and two MBL-1 C57BL/6J chimaeras gave rise to agouti progeny confirming that these ES cells can contribute to the formation of germ cells.

The present invention also relates to chimaeric animals generated by known techniques using the ES cells contemplated herein. These ES cells may be isolated from animal embryos and/or maintained *in vitro* according to the subject invention. Furthermore, genetically manipulated ES cells may be passaged in LIF and used to make chimaeric animals. For example, genetically manipulated ES cells containing a retrovirus vector (N-TK527; derived from pXT1; C.A. Boulter and E.F. Wagner, (1987) Nucl. Acids Res. 15:7194) encoding genes for neomycin resistance and c-src^{527} were propogated in the presence of LIF but in the absence of feeder cells for over 20 passages. These cells still retained the ability to differentiate as judged by the formation of normal chimaeras following introduction of these cells into preimplantation embryos by blastocyst injection.

Further details of the use of LIF in accordance with the present invention will be apparent from the following Examples.

EXAMPLE 1

This example sets out the steps used to maintain ES cells *in vitro* in LIF, and to generate chimaeric mice using ES cells so passaged.

Step 1: Propagation *in vitro*:

The ES cells used were the D3 (Doetschman, T.C. et.al. (1985) J.Embryol.Exp.Morphol. 87:27-45) the EKcs-1 (previously known as CS1) (Wagner, E.F. et.al. (1985) Cold Spring Harbor Symp.Quant.Biol. 50:691-700) and the HD5 (C. Stewart, unpublished) ES cell lines isolated from 129 SV He blastocysts and the CBL63 (R.Kemler, unpublished) ES cells isolated from C57BL/6J blastocysts. Prior to

culture in LIF, the D3 and CBL63 cells were maintained in Dulbecco modified Eagles medium with 15% (v/v) foetal calf serum on a feeder layer of primary embryo fibroblasts, and the EKcs-1 and HD5 ES cells were maintained in Eagle's medium with 15% (v/v) foetal calf serumand 0.1 mM β-mecraptoethanol, in the presence of medium conditioned by the bladder carcinoma cell line 5637 (ATCC No.HTB9).

The ability of recombinant LIF to maintain the stem cell phenotype of ES cells was demonstrated by transferring ES cells of the lines D3 and HD5 into normal cell culture medium in the presence of varying concentrations of purified yeast-derived recombinant human LIF (hereafter referred to as rY-HLIF), or E.coli derived recombinant mouse LIF (rE-MLIF) (previously disclosed in International Patent Application No. PCT/AU88/00093). The results are shown in Figures 1 and 2. In Figure 1A, HDS cells previously maintained in 80% 5637 conditioned medium for eight passages were transferred to culture media containing 0-5,000 units ml^{-1} of purified, recombinant yeast-derived human LIF (H-LIF; see below) (▫-▪) or purified, recombinant E. coli-derived mouse LIF (M-LIF; see below) (o-o). HD5 cells maintained in medium containig 1,000 units ml^{-1} H-LIF for a further 13 passages were then transferred to 0-1,000 units ml^{-1} M-LIF (●-●). In Figure 1B, D3 cells maintined on mouse embryo fibroblasts for 10 passages were transferred to media containing 1,000-5,000 units ml^{-1} H-LIF and after a further 7 or 15 passages the cells were transferred into media containing 0-5,000 units ml^{-1} of H-LIF (▪-▪) or 0-1,000 units ml^{-1} M-LIF (●-●) respectively. Figure 2 shows ES cell morphology in the presence of recombinant LIF. HD5 ES cells cultured in the presence of 80% 5637 conditioned medium were assayed for the ability of purified recombinant LIF to maintain the stem-cell

phenotype by transfer to media containing 1,000 units ml^{-1} M-LIF (A), or to normal culture media (B). After seven days, the colonies were stained with Giemsa. Compact stem-cell colonies could be distinguished from diffuse differentiated colonies. D3 cells maintained in H-LIF for 15 passages were assayed for the ability to differentiate by transfer into media containing 1,000 units ml^{-1} M-LIF (C) or normal culture media (D). Immunofluorescence of the cells in the two D3 colony types was carried out using the ECMA-7 monoclonal antibody which recognizes a stem cell-specific cell-surface antigen. Cell-surface-specific immunofluorescence was detected on over 90% of the cells maintained in media containing 1,000 units ml^{-1} recombinant LIF (E) but on less than 1% of the cells maintained in normal culture media (F). The field of view shown in (F) contains 21 cells.

Figures 1 and 2 indicate that over 90% of the ES cells maintained in 1000-5000 units/ml rY-HLIF or rE-MLIF retained their stem cell phenotype. In contrast, ES cells maintained in normal culture medium differentiated over a period of 3-6 days. The different concentrations of rY-HLIF or rE-MLIF used did not result in any noticeable change in cell number after 6 days in culture, indicating that there is no selection for a specific subpopulation able to grow in LIF. Similar results have been obtained using yeast-derived rMLIF also disclosed in International Patent Application No. PCT/AU88/00093. The data in Figure I indicate that human LIF acts on mouse ES cells, as previously described for the action of human LIF on M1 myeloid leukaemic cells (Gough,N.M. et.al. (1988) Proc.Natl.Acad.Sci.USA 85: 2623-2627). The data in Figure I also indicate that the action of LIF on ES cells is independent of glycosylation, as previously described for the action of LIF on M1 myeloid leukaemic cells.

Four ES cell lines, D3, EKcs-1, CBL63 and HD5, were maintained in medium containing 1000-5000 u/ml rY-HLIF for up to 22 passages (10 weeks or approximately 100 generations). Long-term maintenance of the ES cells in rY-HLIF did not noticeably alter the growth characteristics of the cells. Furthermore, reduction or removal of the LIF from the culture medium resulted in the differentiation of the ES cells with similar kinetics to those explanted directly from bladder carcinoma 5637 conditioned medium or a feeder layer of mouse fibroblasts (for example, see Figures 1 and 2). The stem cell phenotype of ES cells cultured for multiple passages in the presence of LIF was confirmed by immunofluorescence with the ECMA-7 antibody which recognises a cell-surface stem-cell-specific antigen (Kemler, R. in Progress in Developmental Biology Band 26 Sauer, H. W.ed page 175; Fisher, Stuttgart, 1980); ES cells cultured in the presence of LIF expressed the stem cell marker, whereas in the absence of LIF less than 1% did so (Figure 2).

<u>Step 2: Isolation of ES cell lines</u>:

Murine blastocysts were isolated from 129 Sv He mice at day 4 of development (day 1 = day of plug) into either Dulbecco's or Glasgows modified Eagle's medium with 15% (v/v) foetal calf serum, 0.1mM ß-mercaptoethanol and 1000 units/ml of purified rE-HLIF. ES cell lines were then isolated by two different methodologies.

In the first method the blastocysts were allowed to attach to the culture dish and approximately 7 days later the outgrowing inner cell mass picked, trypsinised and transfered to another culture dish in the same culture media. ES cell colonies appeared 2 - 3 weeks later with between 5 - 7 individual colonies arising from each explanted inner cell mass. The ES cell lines were then

expanded for further analysis. The second method for isolation of ES cell lines used the immunosurgery technique (described in Martin, G. R. (1981) Proc. Natl. Acad. Sci. USA 78:7634-7638) where the trophectoderm cells are destroyed using anti-mouse antibodies prior to explanting the inner cell mass. The efficiency of ES cell lines isolation is shown in Table 1.

Step 3: Generation of Chimaeric Mice:

All the ES cell lines cultured in the absence of feeder cells but in the presence of LIF (referred to in step 1) or directly isolated with the aid of culture medium containing LIF (referred to in step 2) retained the ability to differentiate into multiple cell types following the removal of LIF indicating that these cells have retained their pluripotential phenotype. To confirm their developmental potential, D3 ES cells maintained in LIF for 7-22 passages and MBL-1 ES cells maintained in LIF for 14-17 passages were reintroduced into the embryonic environment by blastocyst injection (as described in Williams et al., (1988) Cell 52:121-131). Blastocysts were isolated from the outbred ICR mouse strain or inbred C57BL/6J mice. The expanded blastocysts were maintained in oil-drop cultures at 4°C for 10 min prior to culture. The ES cells were prepared by picking individual colonies, which were then incubated in phosphate-buffered saline, 0.5 mM EGTA for 5 min; a single cell suspension was prepared by incubation in a trypsin-EDTA solution containing 1% (v/v) chick serum for a further 5 min at 4°C. Five to twenty ES cells (in Dulbecco's modified Eagle's Medium with 10% (v/v) foetal calf serum and 3,000 units/ml DNAase 1 buffered in 20 mM HEPES [pH 8]) were injected into each blastocyst. Blastocysts were transferred into pseudopregnant recipients and allowed to

develop normally. Chimaeric mice were identified by coat markers (Hogan et al., (1986) Manipulating the Mouse Embryo, Cold Spring Harbor, New York). Analysis of the subsequent chimaeric mice revealed that up to approximately 50% of the progeny contained tissues derived from the injected cells (Table 2), with levels of overt chimaerism as high as 90% in individual mice. Furthermore analysis of the organs of four D3-chimaeras confirmed that the ES cells maintained in LIF could contribute extensively to the development of all of the somatic tissues (Table 3).

The male chimaeras were tested for germline transmission of ES derived cells by mating to ICR or C57BL/6J females. Three out of four of the D3-C57BL/6J chimaeras and two out of six of the MBL-1-C57BL/6J chimaeras gave rise to agouti offspring derived from the ES cells cultured in LIF (Table 4).

To test whether genetically altered ES cells could be maintained in culture medium containing LIF, D3 ES cells were infected with a retrovirus vector (N-TK527) expressing the neomycin resistance gene and a c-src gene mutant (c-src^{527}) (protocol for infection is described in Williams et al., (1988) Cell 52: 121-131). The ES cell clones isolated were maintained in culture medium containing LIF for over 20 passages. These genetically modified ES cells retained the ability to form chimaeric mice following reintroduction into the embryonic environment by blastocyst injection (Table 2)

TABLE 1: Isolation of 129 Sv He ES cell lines in media containing rE-HLIF

Methodology	Blastocyst	ICM outgrowing	Number of ES cell lines derived
Explanted	9	9	4
Immunosurgery	11	3	0
Immunosurgery	7	5	2

Murine blastocysts were isolated from 129 Sv He mice at day 4 of development (day 1 = day of plug) into either Dulbecco's or Glasgows modified Eagle's medium with 15% (v/v) foetal calf serum, 0.1mM ß-mercaptoethanol and 1000 units/ml of purified rE-HLIF. The blastocysts were then explanted into the same media and left to attach to the culture dish and the inner cell mass picked dissociated in phosphate-buffered saline, 0.5 mM EGTA for 5 min; a single cell suspension was prepared by incubation in a trypsin-EDTA solution containing 1% (v/v) chick serum and the cells replated in the cell culture medium described above. The characteristic ES cell colonies appeared within 1 - 3 weeks.

Other blastocysts were treated by immunosurgery (as described in Martin, G. R. (1981) Proc. Natl. Acad. Sci. USA 78:7634-7638). The blastocysts were allowed to hatch from the zona pelucida, and then treated with anti-mouse antibodies and destroyed by the addition of complement. The exposed inner cell mass was then left to

attach to a tissue culture dish and again treated with anti-mouse antibodies and complement. Within a few days pluripotential stem cell colonies appeared and were dissociated and trypsinised as described above.

Table 2 Chimaeric mice derived from ES cells cultured in LIF

ES cells	Blastocysts transferred	Pups born	Chimaeras
D3	142	60(42%)	33(55%)
MBL-1	51	33(65%)	16(48%)
D3 N-TK527	42	22(52%)	12(54%)

Table 3

Percentage tissue contributions in individual D3 chimaeric mice

Chimaera	Necropsy age	C	Bl	Sp	P	Li	T	H
D3-1	13d	35	0	35	20	10	20	40
D3-2	14d	40	15	35	30	45	30	50
D3-3	11d	90	50	50	35	50	40	60
D3-4	11d	50	50	50	30	40	40	50

Chimaera	Necropsy age	Lu	G	K	M	B	Sa
D3-1	13d	30	10	35	30	35	20
D3-2	14d	35	20	30	50	50	25
D3-3	11d	45	50	50	70	50	55
D3-4	11d	50	35	50	50	20	30

TABLE 4 Chimaeric demonstrating germline transmission of ES derived cells.

Mice	Chimaerism	Passage no. of D3 cells on feeders	in LIF	Offspring 129 Sv He	C57
775-3	75%	10	16	9	24
778-1	70%	10	22	5	33
778-2	50%	10	22	2	36
778-3	55%	10	22	0	0

SUBSTITUTE SHEET

The following relates to Tables 2, 3 and 4:

D3 and MB1-1 ES cells are derived from 129 Sv He mice (inbred, agouti, homozygous for the glucose phosphate isomerase 1^a allele). The D3 ES cells were originally cultured on primary embryo fibroblasts for 10 passages and then transferred to 1,000-5,000 units/ml recombinant LIF for 7-22 passages. The MB1-1 ES cells were isolated in the absence of feeder cells but in the presence of rE-HLIF these cells were cultured for 14-17 passages. The ES cells were then injected into ICR (outbred, albino) or C57BL/6J (inbred, black) blastocysts which were then transfered into pseudo-pregnant foster mothers. Both the ICR and C57BL/6J mice are homozygous for the glucose phosphate isomerase 1^b allele. Chimaeric pups were identified by coat pigmentation (only foster mothers which became pregnant were counted in estimating the number of progeny). Tissue chimaerism was estimated using glucose phosphate isomerase strain differences. The extent of tissue chimaerism was determined in two D3-ICR (numbers 1 and 2) and two D3-C57BL/6J chimaeras (numbers 3 and 4). Tissues analysed: C, coat; Bl, blood; Sp, spleen; P, pancrease; Li, liver; T, thymus; H, heart; Lu, lungs; G, gonads; K, kidneys; M, muscle; B, brain; Sa, salivary gland. Male chimaeras were mated to ICR or C57BL/6J mice and offspring identified by coat pigmentation.

EXAMPLE 2

This example sets out the steps used to document specific high affinity receptors on ES and EC cells. Accompanying Figure 3 shows binding of ^{125}I-LIF to ES cells EKcs-1 and EC cells F9 and PCC3-A (Jakob, J. et.al. (1973) Ann.Microbiol.Inst.Pasteur, 124B: 269-282). In relation to Figure 3, (A), Scatchard analysis of ^{125}I-labelled LIF binding to F9 (□), EKcs-1 (●), PCC3A-1 (■) and M1 (○) cells. Saturation curves for binding were analysed by the method of Scatchard by plotting the amount of LIF specifically bound (defined as the difference between binding observed in the absence and presence of excess unlabelled LIF) versus the ratio of bound to free LIF. Free LIF values were adjusted for the percent of ^{125}I-labelled LIF capable of binding specifically to LIF receptors, in this experiment determined to be 75%. The apparent dissociation constant for the interaction of LIF with its receptor was determined from the slopes of the curves and the receptor number from their intercepts with the ordinate. Results in (A) were standardized to 5×10^6 cells per point and the mean of duplicate points are shown and curves were fitted using the Ligand program. (B), Autoradiography of F9 EC cells labelled with ^{125}I-labelled LIF. (C), Quantitation of silver grains on F9 EC cells after binding of ^{125}I-labelled LIF.

Purified recombinant (yeast-derived) human LIF (rY-HLIF) was radioactively labelled on tyrosine residues as described previously (Hilton, D.J.et.al.(1988) Proc.Natl.Acad.Sci. USA, 85:5971-5975) producing ^{125}I-LIF with a specific radioactivity of approximately 1.2×10^7 cpm/pmole. ^{125}I-LIF (2×10^3 - 5×10^5 cpm) was incubated with 1-4 x 10^6 target cells with or without at least 100-fold molar excess of unlabelled LIF, in a total volume of 100µl for 4 hours on ice. Cell-associated and free ^{125}I-LIF were separated by centrifugation through foetal

calf serum (Nicola, N.A. and Metcalf, (1986) D. J.Cell Physiol. 128:160-188). Specific cell-associated ^{125}I-LIF was determined by cold competition.

Figure 3 illustrates the specific saturable and high affinity binding of ^{125}I-LIF to the ES cells EKcs-1 and the EC cells PCC3-A and F9. The number of LIF receptors per cell derived from these Scatchard plots were 295, 190 and 330, respectively, with apparent dissociation constants at 4°C of approximately 90 pM. This compares with the M1 cell line, a LIF-responsive monocytic leukaemia, which displays 50-200 LIF receptors/cell with an apparent dissociation constant of 50-150 pM. All other ES and EC cells tested - D3, NG2, PC13 and P19 - bound similar levels of LIF (data not shown).

The binding of ^{125}I-LIF to M1 cells, EKcs-1 and PCC3-A was also found to be in competition with unlabelled recombinant and native murine and human LIF, but not with the range of other hormones and factors, (including several which act on embryonic cells): insulin, IGF-I, IGF-II, acidic and basic FGF, TGFβ, TNFα, TNFβ, NGF, PDGF, EGF, IL-1, IL-4, GM-CSF, G-CSF, Multi-CSF and erythropoietin.

CLAIMS

1. A method for the isolation of embryonic stem (ES) cells from animal embryos in vitro which method comprises deriving and maintaining said embryos in culture medium containing an effective amount of leukaemia inhibitory factor (LIF) for a time and under conditions sufficient for the development of said ES cells.

2. The method according to claim 1 wherein the culture medium is free of feeder cells.

3. The method according to claims 1 or 2 wherein the animal embryos are derived from humans, mice, birds, sheep, pigs, cattle, goats or fish.

4. The method according to claim 3 wherein the animal embryos are derived from mice.

5. The method according to claims 1 or 2 wherein the culture medium is Eagle's medium or modifications thereof or equivalents thereto.

6. The method according to any one of the preceding claims wherein the LIF is recombinant LIF.

7. The method according to claim 6 wherein the LIF is recombinant human or murine LIF.

8. The method according to claim 7 wherein LIF is added to the culture medium at a concentration of from 10 to 1,000,000 units/ml.

9. The method according to claim 8 wherein the LIF is added to the culture medium at a concentration of from 100 to 100,000 units/ml.

10. The method according to claim 9 wherein the LIF is added to the culture medium at a concentration of from 500 to 10,000 units/ml.

11. A method according to any one of the preceding claims wherein the effective time is from 1 day to 20 weeks.

12. The method according to claim 11 wherein the effective time is from 1 to 8 weeks.

13. A method for maintaining animal embryonic stem (ES) cells <u>in vitro</u> while retaining their pluripotential phenotype which process comprises culturing said cells in a culture medium containing an effective amount of leukaemia inhibitory factor (LIF) under conditions sufficient to maintain said cells.

14. The method according to claim 13 wherein the culture medium is free of feeder cells.

15. The method according to claim 13 or 14 wherein the animal ES cells are derived from humans, mice, birds, sheep, pigs, cattle, goats or fish.

16. The method according to claim 15 wherein the animal ES cells are derived from mice.

17. The method according to claim 13 or 14 wherein the culture medium comprises Eagle's medium or modifications thereof or equivalents thereto.

18. The method according to any one of claims 13 to 17 wherein the LIF is recombinant LIF.

19. The method according to claim 18 wherein the LIF is recombinant murine or human LIF.

20. The method according to claim 19 wherein the recombinant LIF is added to the culture medium at a concentration of from 10 to 1,000,000 units/ml.

21. The method according to claim 20 wherein the recombinant LIF is added to the culture medium at a concentration of from 100 to 100,000 units/ml.

22. The method according to claim 21 wherein LIF is added to the culture medium at a concentration of from 500 to 10,000 units/ml.

23. Embryonic stem (ES) cells derived from animal embryos *in vitro* isolated by deriving and maintaining said embryos in culture medium, said culture medium containing an effective amount of leukaemia inhibitory factor (LIF) for a time and under conditions sufficient for the development of said ES cells.

24. The ES cells according to claim 23 wherein the culture medium is free of feeder cells.

25. The ES cells according to claim 23 or 24 derived from human, mouse, bird, sheep, pig, cattle, goat or fish embryos.

26. The ES cells according to claim 25 derived from mouse embryos.

27. A chimaeric animal or transgenic progeny thereof generated using ES cells which have been isolated from animal embryos according to claim 1.

28. A chimaeric animal or transgenic progeny thereof generated using animal ES cells which have been maintained <u>in vitro</u> according to the method of claim 13.

29. The chimaeric animal or transgenic progeny thereof according to claim 27 or 28 wherein said animal is a mouse.

30. The chimaeric animal or transgenic progeny thereof according to claims 27 or 28 or 29 wherein the ES cells contain additional genetic material inserted therein.

1/3

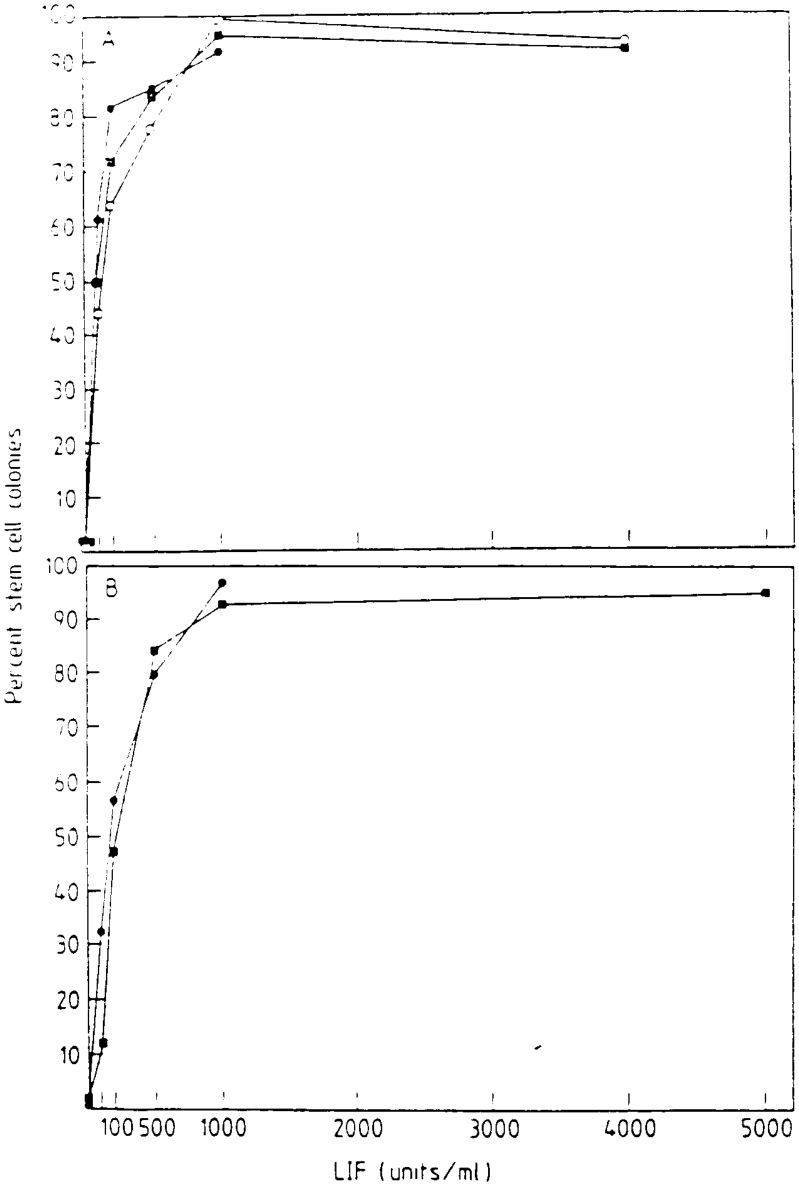
A
B
Percent stem cell colonies
10
20
30
40
50
60
70
80
90
100
100 500
1000
2000
3000
4000
5000
LIF (units/ml)

Fig.1.

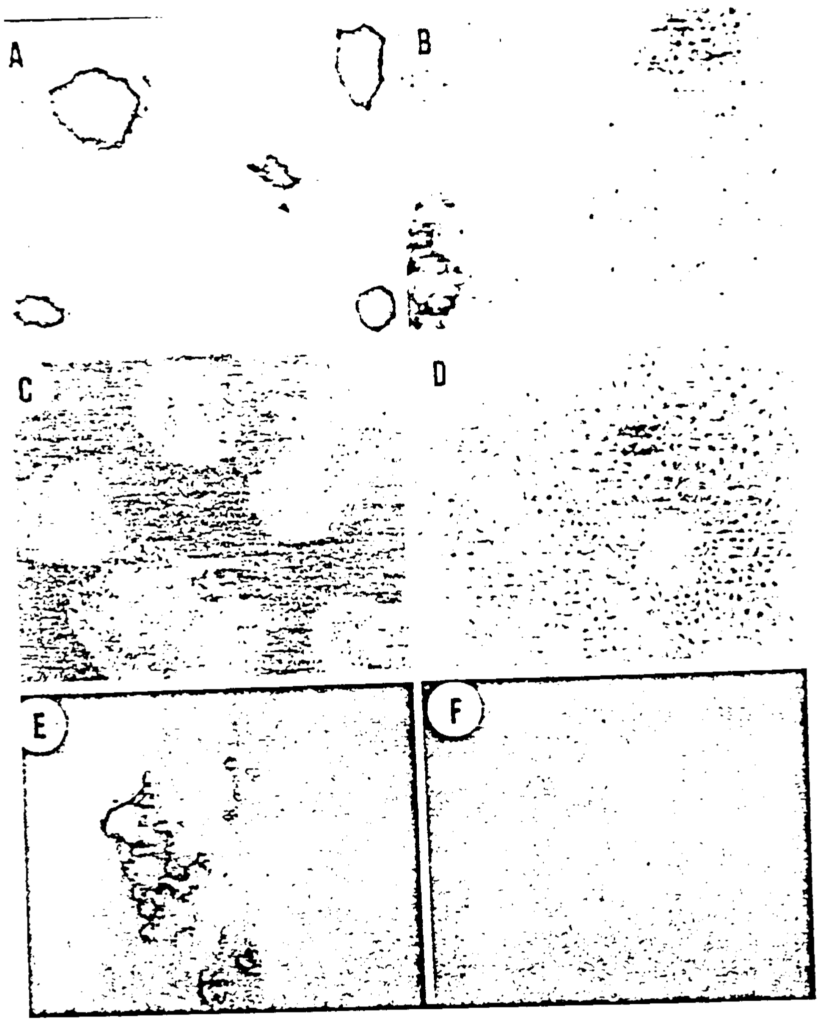

Fig.2.

3/3

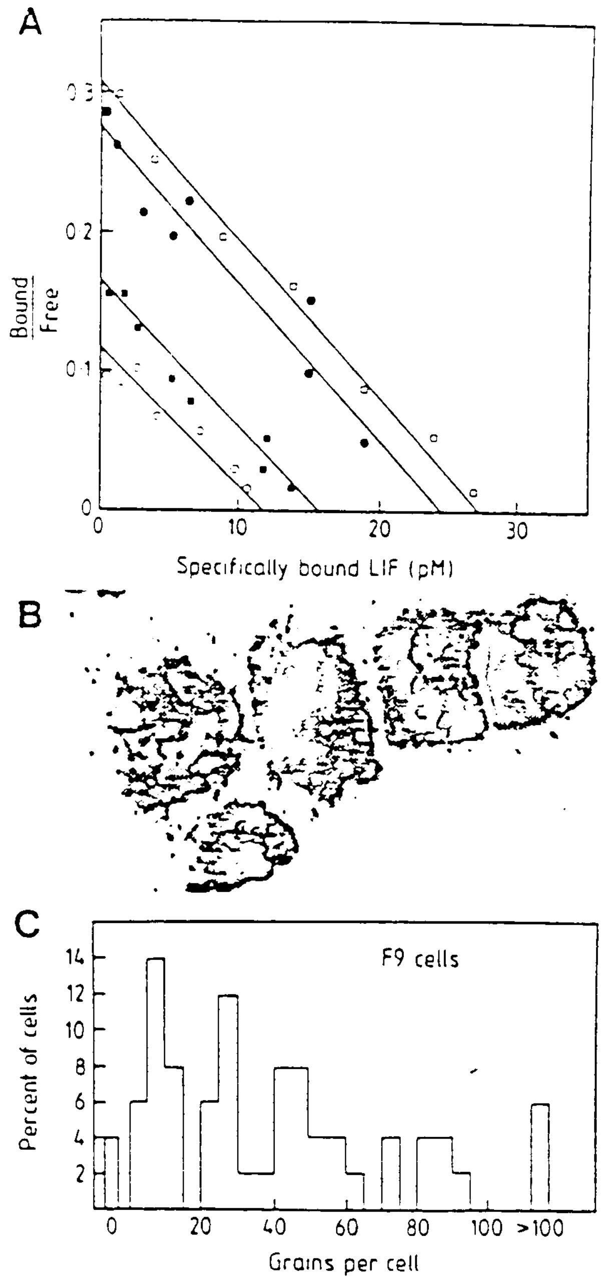
A
0.3
0.2
0.1
0
Bound/Free
0
10
20
30
Specifically bound LIF (pM)
B
C
F9 cells
14
12
10
8
6
4
2
Percent of cells
0
20
40
60
80
100
>100
Grains per cell

Fig.3.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/AU 89/00330

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 6

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl.[4] C12N 5/00, 1/38, 15/00, A01K 67/00

II. FIELDS SEARCHED

Minimum Documentation Searched 7

Classification System	Classification Symbols
IPC	WPI, WPIL, USPA Keywords: "Embryonic stem cells, ES cells, Leukaemia inhibitory factor, LIF"

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 8

AU: C12N 1/38, 5/00, 5/02, A61K 67/00, 67/02
Chemical Abstracts: Keywords: as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category*	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No 13
P,X	Williams, R.L. et al. "Myeloid Leukaemia Inhibitory factor maintains the developmental potential of embryonic stem cells", Nature, Bolume 336, issued 15 December 1988, (USA), see pages 684 to 687.	1 to 30
P,X,Y	Smith, A.G. et al. "Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides", Nature, Volume 336 issued 15 December 1988, (USA), see pages 688 to 690.	1 to 30
P,Y	Heath, J.K. and A.G. Smith, "Regulatory factors of embryonic stem cells", Journal of Cell Science, Supplement, Volume 10, issued 1988, (The Company of Biologists Limited 1988, Great Britain), see pages 257 to 266.	1 to 30

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
2 November 1989 (02.11.89)	10 November 1989
International Searching Authority	**Signature of Authorized Officer**
Australian Patent Office	J. ASHMAN

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category*	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Smith, A.G. and M.L. Hooper, 'Buffalo Rat Liver Cells Produce a Diffusable Activity which Inhibits the Differentiation of Murine Embryonal Carcinoma and Embryonic Stem Cells', Developmental Biology, Volume 121, issued 1987 (Academic Press, Inc, USA), see pages 1 to 9	1 to 30
A	Chemical Abstracts, Volume 105, issued 18 August 1987, (Columbus, Ohio, U.S.A.) R.H. Lovell - Badge et al. 'Transformation of embryonic stem cells with the human type-III collagen gene and its expression in chimeric mice', see page 169, column 2, abstract no. 5563-6, Cold Spring Harbour Symp. Quant. Biol. 1985, 50 (Mol. Biol. Dev.), 707-11.	
A	Chemical Abstracts, Volume 108, issued 14 March 1988, (Columbus, Ohio, U.S.A.) D.P. Gearing et al. 'Molecular cloning and expression of cDNA encoding a murine myeloid leukemia inhibitory factor (LIF)', see page 159, column 1, abstract no. 88918m, EMBO J., 1987, 6 (13), 3995-4002.	
P,A	Chemical Abstracts, Volume 109, issued 15 August 1988, (Columbus, Ohio, U.S.A.), D.J. Hilton et al. 'Resolution and purification of three distinct factors produced by Krebs ascites cells which have differentiation - Inducing activity on murine myeloid leukemia cell lines', see page 522, column 2, abstract no. 52951s, J. Biol. Chem. 1988, 263 (19), 9238-43.	